

Local Delivery of Marrow-Derived Stromal Cells Augments Collateral Perfusion Through Paracrine Mechanisms

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Background—Bone marrow cell therapy is reported to contribute to collateral formation through cell incorporation into new or remodeling vessels. However, the possible role of a paracrine contribution to this effect is less well characterized.

Methods and Results—Murine marrow-derived stromal cells (MSCs) were purified by magnetic bead separation of cultured bone marrow. The release of vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), placental growth factor (PIGF), and monocyte chemoattractant protein-1 (MCP-1) was demonstrated by analysis of MSC conditioned media (MSC-CM). MSC-CM enhanced proliferation of endothelial cells and smooth muscle cells in a dose-dependent manner; anti-VEGF and anti-FGF antibodies only partly attenuated these effects. Balb/C mice ($n=10$) underwent distal femoral artery ligation, followed by adductor muscle injection of 1×10^6 MSCs 24 hours later. Compared with controls injected with media ($n=10$) or mature endothelial cells ($n=8$), distal limb perfusion improved, and mid-thigh conductance vessels increased in number and total cross-sectional area. MSC injection improved limb function and appearance, reduced the incidence of auto-amputation, and attenuated muscle atrophy and fibrosis. After injection, labeled MSCs were seen dispersed between muscle fibers but were not seen incorporated into mature collaterals. Injection of MSCs increased adductor muscle levels of bFGF and VEGF protein compared with controls. Finally, colocalization of VEGF and transplanted MSCs within adductor tissue was demonstrated.

Conclusions—MSCs secrete a wide array of arteriogenic cytokines. MSCs can contribute to collateral remodeling through paracrine mechanisms. (*Circulation*. 2004;109:1543-1549.)

Key Words: cells, bone marrow ■ cells, stromal ■ angiogenesis

Several bone marrow subpopulations, such as endothelial progenitor cells and marrow stromal cell fraction (marrow-derived stromal cells [MSCs]), may be able to differentiate into 1 or more of the cellular components of the vascular bed.¹⁻³ Thus, therapeutic delivery of bone marrow donates cells with potential to incorporate into new or remodeling blood vessels. However, the magnitude of incorporation of bone marrow-derived cells into vascular structures varies between studies. Although >50% of capillaries containing transplanted cells have been reported, only a single transplanted cell in the circumference of the vessel is required for it to be counted as a positive vessel.⁴ Furthermore, other studies have reported small numbers of positive vessels, despite impressive improvements in perfusion.⁴⁻⁶ Taken together, these data suggest that other mechanisms may contribute to the improved collateral perfusion observed after stem cell therapy in various models of ischemia.

MSCs play an important supportive role in the marrow microenvironment, mediated partly through cell-to-cell contact but importantly also via paracrine mechanisms involving

release of cytokines that exert effects on surrounding cells. Therefore, the present study sought to examine the hypothesis that local delivery of MSCs augments collateral perfusion in a murine hindlimb ischemia model and that these effects are mediated by paracrine mechanisms rather than by cell incorporation.

Methods

MSC Harvest, Culture, and Isolation

Bone marrow was harvested by flushing the tibiae and femurs of Balb/C mice with DMEM supplemented with 10% fetal bovine medium (DM-10). The pooled marrow was plated in DM-10 supplemented with 1% penicillin-streptomycin and cultured for 72 hours. Nonadherent cells were washed off, and adherent cells expanded until confluent (≈ 7 to 10 days).

MSCs were purified from the heterogeneous cultured cells. The CD34-/CD45- fraction was isolated by labeling with fluorescein isothiocyanate-labeled anti-CD34 antibody (Pharmingen), simultaneous incubation with anti-fluorescein isothiocyanate and anti-CD45 magnetic beads (Miltenyi Biotec), and passage through a magnetic column. Subsequently, the bead-negative and bead-positive populations were cultured separately. The bead-negative

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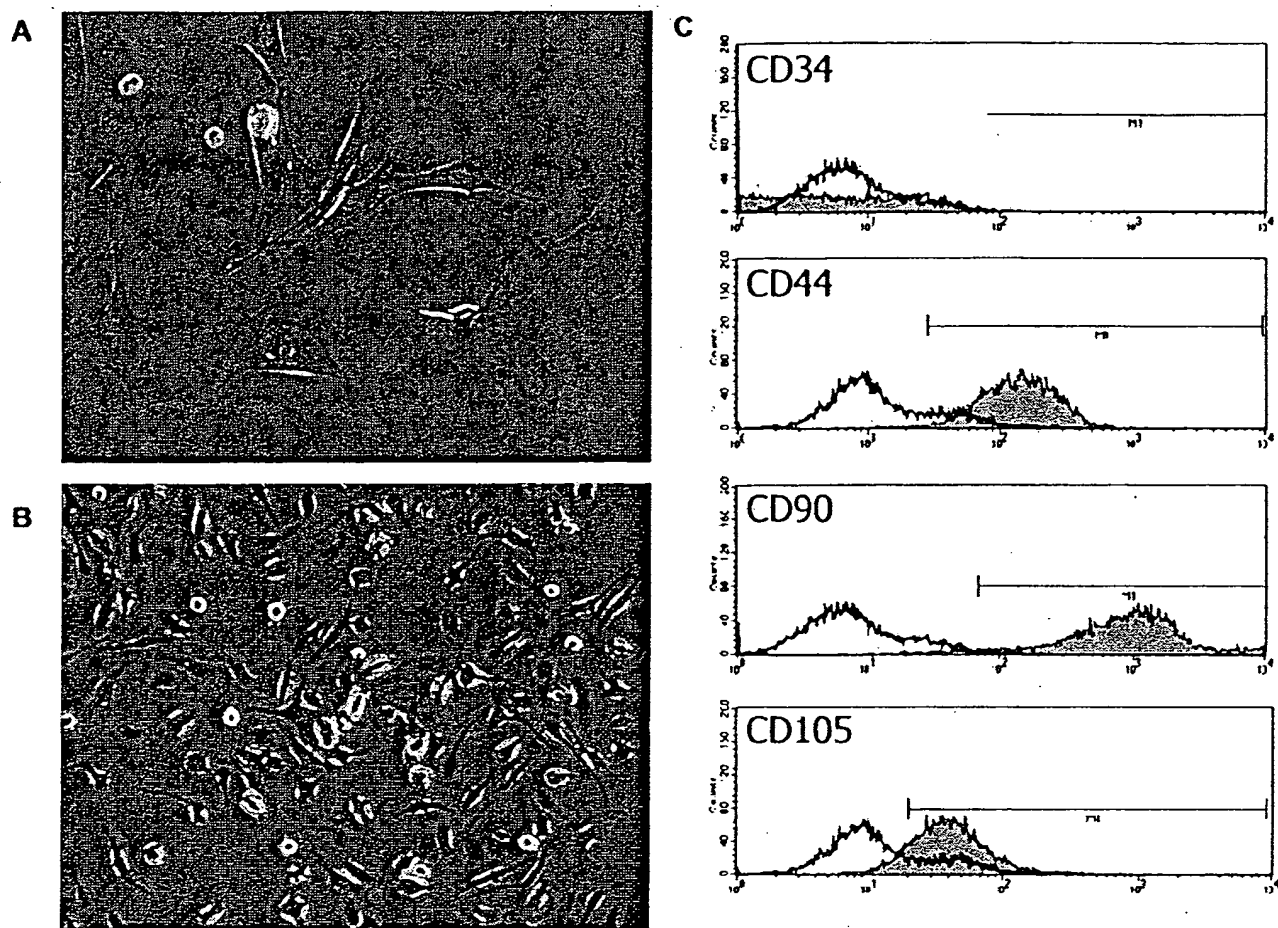


Figure 1. A, CD34⁻/CD45⁻ cells show fibroblastic morphology typical of MSCs. B, CD34⁺/CD45⁺ cells show spherical morphology consistent with lymphohematopoietic cells. C, FACS analysis of murine MSCs. Cells were uniformly negative for CD34 and positive for CD44 (95±0.6%), CD90 (99.1±0.1%), and CD105 (89±2.1%), markers associated with MSCs.

population demonstrated typical MSC fibroblastic morphology, whereas the bead-positive population consisted of small, spherical cells consistent with lymphohematopoietic cells (Figure 1A and 1B). Fluorescence-activated cell sorter (FACS) analysis demonstrated that the bead-negative cells did not express the surface markers CD31, CD34, CD45, and CD117 but did express high levels of CD44 (95±0.6%), CD90 (99.1±0.1%), and CD105 (89±2.1%), in agreement with previous published data on MSC cell surface markers (Figure 1C).⁷

Murine Endothelial and Smooth Muscle Cell Harvest

To isolate mouse aortic endothelial cells (MAECs), thoracic aortas (n=10) were cut into 1- to 2-mm rings after adventitial removal and incubated with 0.25% trypsin for 20 minutes. Floating cells were harvested and cultured in medium-199 supplemented with 10% FBS (M-10). Cells were uniformly positive for factor VIII. Smooth muscle cells (SMCs) were isolated with the use of a modification of a previously described protocol.⁸ Briefly, after MAECs were collected as described above, collagenase in Hanks' balanced salt solution (1 mg/mL) was added and incubated in 37°C for up to 3 hours with gentle agitation every 15 to 30 minutes. Floating cells were harvested, washed, and resuspended in M-10. Cells stained uniformly for smooth muscle actin. Passages 3 to 8 were used for experiments.

Conditioned Media Collection and Analysis

For enzyme-linked immunosorbent assay (ELISA), media were collected from plates of MAECs or MSCs (MSC conditioned media

[MSC-CM]) after 24 hours of culture and analyzed by sandwich ELISA kits (vascular endothelial growth factor [VEGF], basic fibroblast growth factor [bFGF], placental growth factor [MCP-1], and placental growth factor [PlGF]) according to the manufacturer's directions. As a control, basal media were also analyzed. ELISA values were corrected for total cell protein.

Endothelial Cell and SMC Proliferation Assay

MAECs or SMCs (1×10⁴ per well) were cultured in varying dilutions of MSC-CM or control wells of DM-10. When indicated, blocking antibodies to VEGF (10 µg/mL, Sigma) and/or blocking antibodies to bFGF (5 µg/mL, Sigma) were added to the MSC-CM. Cultures were continued for 72 hours, after which the cells were recovered and counted with a Coulter counter. Data are reported as the mean percent change in proliferation compared with control.

Animal Surgery and Cell Delivery

All animal procedures were approved by the institutional animal care and use committee. Under sedation, 12-week-old Balb/C mice (Jackson Laboratories, Bar Harbor, Me) underwent distal femoral artery ligation to create unilateral hindlimb ischemia. In preliminary studies, MSC injection immediately after femoral ligation failed to improve flow recovery. Therefore, in the present study, MSC injection was delayed by 24 hours to allow the mice to recover from surgery. MSCs (1×10⁶ cells in 250 µL volume; n=10), MAECs (1×10⁶ cells in 250 µL volume; n=8), or media alone (n=10) were injected in 6 sites in the right adductor muscle adjacent to and within 1 mm proximal or distal to the ligation site.

In Vivo Assessment of Limb Perfusion, Function, and Ischemic Damage

Blood flow recovery between mid-calf and mid-foot regions was measured with laser-Doppler perfusion imaging (LDPI) (Moor Instruments). For consistent measurements, imaging was performed after limb hair removal and after heating to 37°C to minimize temperature variation. Calculated perfusion is expressed as a ratio of the ischemic to normal limb.⁹ Previous data suggest a close linear relationship between recovery of LDPI perfusion and remodeling of adductor collateral vessels.¹⁰ A semiquantitative functional assessment of the ischemic limb was performed by a blinded observer using a modification of a clinical score (0=toe flexion, 1=foot flexion, 2=no dragging but no plantar flexion, 3=foot dragging).¹¹ Ischemic damage was also scored (0=no change, 1=mild discoloration, 2=moderate/severe discoloration, 3=necrosis, 4=amputation).

Western Blotting

Muscle samples were harvested 24 hours (for hypoxia-inducible factor-1 α [HIF-1 α] assay) or 7 days (for VEGF and bFGF assays) after surgery. Proteins were separated with the use of SDS-PAGE gels (10%) and incubated with antibodies to HIF-1 α (1:800, Santa Cruz), VEGF (1:1000, Chemicon), bFGF (1:1000, Santa Cruz), or α -tubulin (1:2000, Santa Cruz). Relative quantification of proteins was determined with the use of Imagegauge software (Fuji Photo Film Co).

MSC Labeling and Tracking

Preliminary studies determined that 99% of MSCs were transduced with an adenovirus containing a reporter transgene at a multiplicity of infection (MOI) of 150 (data not shown). To track protein expression, cells were incubated with Ad.GFP or Ad. β -galactosidase at MOI of 150 for 2 hours and immediately injected into the adductor muscle (24 hours after surgery). To follow the fate of injected green fluorescent protein (GFP)+MSCs, sections of adductor and calf muscle were examined with the use of a Nikon inverted fluorescent microscope. To follow the fate of β -gal+/MSCs, sections were developed with an X-gal kit (Invitrogen). For demonstration of vessels, adductor muscle sections were stained with a goat anti-mouse platelet-endothelial cell adhesion molecule (PECAM) antibody (Santa Cruz).

Immunofluorescence and Histological Analysis

For colocalization of MSCs and VEGF, MSCs were incubated *in vivo* with carboxyfluorescein diacetate succinimidyl esters (CFSE) (Molecular Probes) and injected into adductor muscle. At day 7, cryostat sections of adductor muscle were fixed in methanol, blocked with 10% FBS for 30 minutes, and incubated with goat anti-mouse VEGF antibody (1:500, Santa Cruz) and then with phycoerythrin-coupled anti-goat antibody (Santa Cruz). Adductor muscle sections were stained with van Gieson's solution, and conductance arteries, identified by the presence of a continuous internal elastic laminae and muscle spindles, were counted. Total cross-sectional area was calculated with the use of Image-Pro software, with the smallest internal luminal distance measured as the radius. For collagen analysis, calf muscle sections were fixed in formalin and stained with 0.1% Sirius red. Collagen volume fraction was determined by measuring the percentage of the total area of stained tissue within a given field.

Statistical Analysis

All results are presented as mean \pm SEM. Statistical significance was evaluated with an unpaired Student *t* test for comparison between 2 groups or with ANOVA for comparison and contrast between multiple groups. A probability value of <0.05 was considered significant.

Results

MSC-CM Cytokine Content and Effect on Cell Proliferation

Previous studies demonstrated the importance of VEGF and bFGF in mediating tissue responses to ischemia.¹² Therefore, to characterize MSC release of these and other cytokines, we collected MSC-CM for 24 hours. With the use of ELISA, the release of VEGF (375 pg/ μ g protein from MSC-CM versus 34 pg/ μ g protein from MAECs; $P<0.01$), bFGF (2320 versus 25 pg/ μ g; $P<0.001$), PlGF (119 pg/ μ g versus nondetectable; $P<0.05$), and MCP-1 (150 versus 4 pg/ μ g; $P<0.05$) in MSC-CM was demonstrated (Figure 2A). Cytokines were not detected in basal culture media.

To examine whether MSC-CM exhibited biological effects, a series of endothelial cell and SMC proliferation assays were performed. MSC-CM significantly enhanced endothelial cell proliferation over control in a dose-responsive manner (Figure 2B). Anti-VEGF antibody partially attenuated the effects of MSC-CM, although there was still a significant mitogenic effect over control (450% over control without antibody versus 190% over control with antibody; $P<0.001$; Figure 2B). In contrast, anti-bFGF antibody had a smaller effect on endothelial cell proliferation (450% without antibody versus 375% with antibody; $P<0.05$). Anti-VEGF antibody had little effect on SMC proliferation, although anti-bFGF antibody significantly impaired SMC proliferation (82% over control without antibody versus 31% with antibody; Figure 2C). Similar to endothelial cell proliferation, there remained a significant increase in cell number over control despite blocking VEGF and bFGF, implying the mitogenic influence of multiple MSC-CM cytokines.

Analysis of HIF-1 α Induction *In Vivo*

HIF-1 α protein level, a sensitive marker of ischemia, was measured to assess adductor muscle injection site ischemia. Quantitative analysis of the HIF Western blot showed a 30-fold increase in the calf HIF protein at day 1 and a 15-fold increase in the calf HIF protein at day 3 over control (Figure 3). There was no HIF protein demonstrated in the adductor samples. These data imply no or minimal ischemia at the injection site.

Hindlimb Blood Flow and Functional Recovery

All animals survived surgery and reached the 21-day end point. In mice receiving media or MAECs, flow returned to approximately 50% of the nonischemic limb by day 14, with no difference in flow recovery between the 2 groups. In contrast, in MSC-treated mice, there was a significant improvement in flow (Figure 4A), and a dose-response relationship was demonstrated (Figure 4B). In mice receiving MSCs, there was an increase in the number of mid-thigh arteries (6.6 versus 4.1 in those mice receiving media [$P<0.05$] versus 4.3 in those mice receiving MAECs [$P<0.05$]; Figure 4D). Similarly, total arterial cross-sectional area was increased in those mice compared with control (10 453 versus 6723 μ m² [$P<0.05$] versus 5125 μ m² [$P<0.05$], respectively).

Improved flow recovery led to improved hindlimb appearance and function. Mice receiving media or MAECs experi-

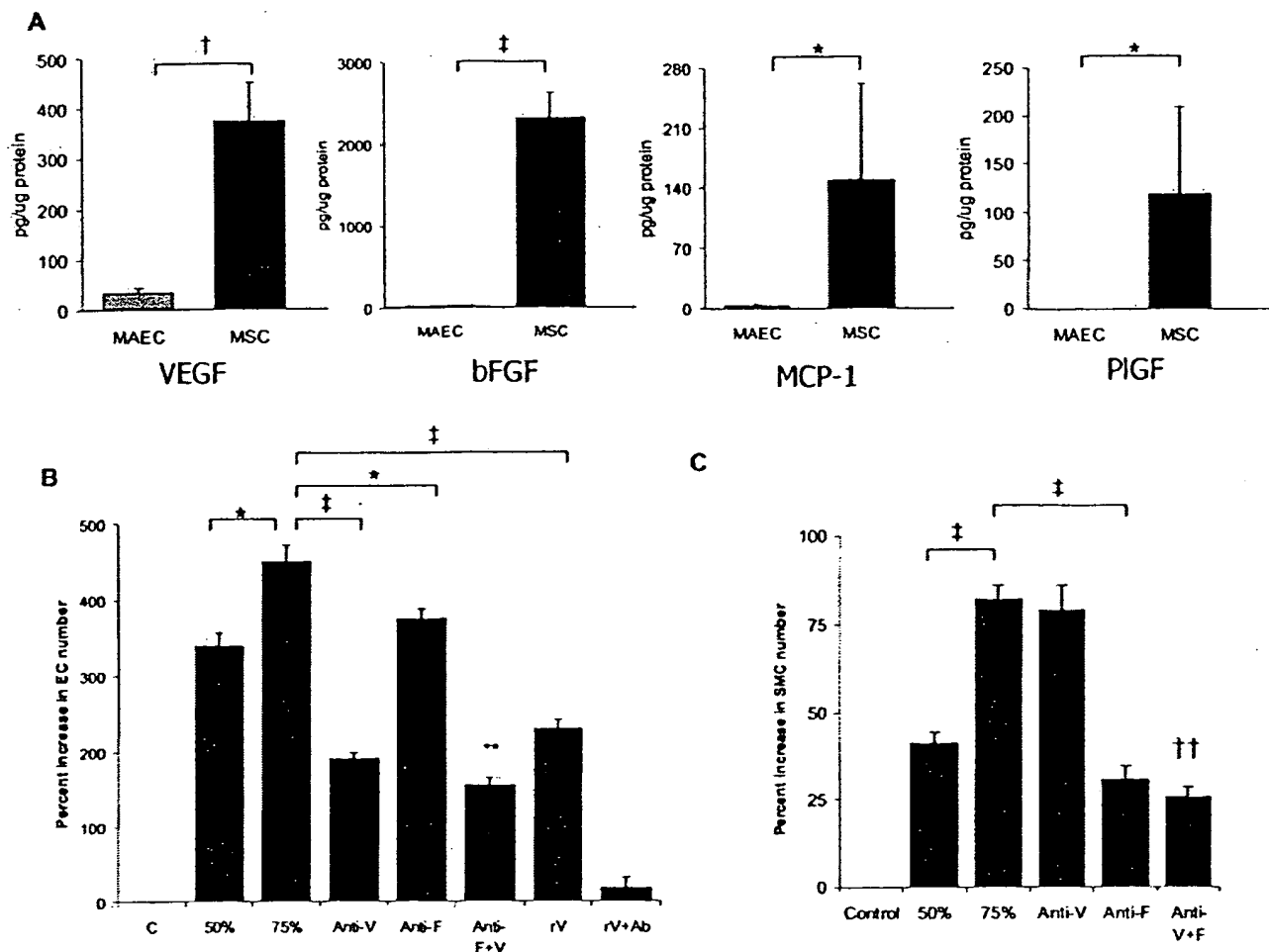


Figure 2. A, In vitro release of VEGF, bFGF, PIGF, and MCP-1 from MSCs and MAECs ($P < 0.05$, $\dagger P < 0.01$, $\ddagger P < 0.001$). B, In vitro biological effects of 50% and 75% dilutions of MSC-CM on MAEC proliferation over control media with or without blocking VEGF (anti-V) and FGF (anti-F) antibodies (Ab). Also shown is rVEGF (rV) with or without anti-V as a control ($P < 0.05$, $\dagger P < 0.001$, $**P < 0.0001$ over control). C, In vitro biological effects of 50% and 75% dilutions of MSC-CM on SMCs over control media with or without blocking antibodies ($\dagger P < 0.001$, $\ddagger P < 0.05$ over control). EC indicates endothelial cells.

enced severe ischemic damage (ischemic score, 2 ± 0.5 versus 2 ± 0.62 , respectively; $P = \text{NS}$), resulting in a 50% incidence of auto-amputation by day 21. However, mice receiving MSCs displayed less ischemic damage (ischemic score, 0.2 ± 0.15 ; $P < 0.05$ for both control group comparisons), with a 10% auto-amputation rate (Figure 5A). Similarly, in mice receiving media or MAECs, significant impairment of function remained at day 21 (1.75 ± 0.24 in media group versus 2.4 ± 0.3 in MAEC group; $P = \text{NS}$). However, in the MSC group, ambulatory impairment was less than both control groups (ischemic score, 0.4 ± 0.18 ; Figure 5B).

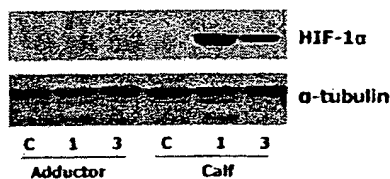


Figure 3. Strong HIF-1 α protein induction in the calf muscle of the hindlimb in which the femoral artery was ligated, and absence of HIF-1 α protein induction in the adductor muscle of the same limb. MSCs were injected into the adductor muscle.

Muscle Atrophy and Fibrosis

Significant calf muscle atrophy was noted in the media- and MAEC-treated mice ($65.2 \pm 6.2\%$ versus $60.3 \pm 6.5\%$, respectively; $P = \text{NS}$). MSC transplantation significantly attenuated this tissue loss ($36.1 \pm 8.9\%$; $P < 0.05$ versus media; Figure 5C). Muscle fibrosis was more pronounced in the media and MAEC groups compared with MSC ($28.8 \pm 2.0\%$ versus $29.2 \pm 2.0\%$ versus $14.5 \pm 1.3\%$; $P < 0.001$; Figure 5D). Fiber atrophy and disturbance of normal tissue architecture was also more evident in mice not receiving MSCs (Figure 5E).

MSC Tracking and Protein Expression in Adductor Muscle

To assess MSC viability and distribution, MSCs were transduced ex vivo with an Ad.GFP vector and injected immediately. The subsequent appearance of GFP+ cells implied viability and maintenance of the transcriptional and translational mechanisms. Few fluorescent cells were found in adductor sections taken at day 3. However, strongly fluorescent cells were observed in large numbers by day 7 and persisted through day 14 (Figure 6A, top). By day 21, cell

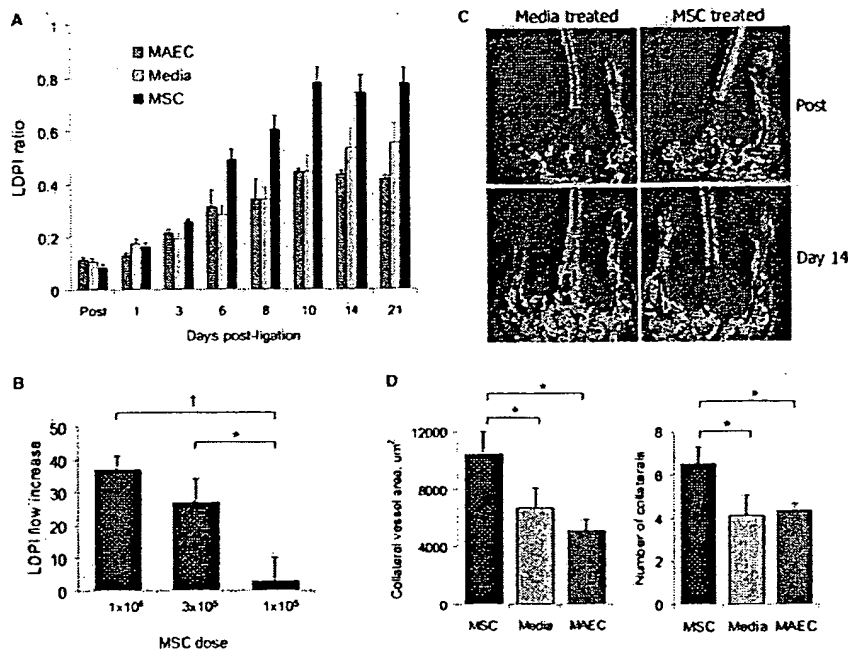


Figure 4. Perfusion analysis after cell treatment. **A**, LDPI expressed as a percentage of the normal limb. Flow recovery in MSC-treated animals was significantly better than that seen in control animals ($P < 0.001$ for MSC trend vs both controls by ANOVA). **B**, Evidence of dose-response relationship. Percent increase in flow over media control at day 21 is shown ($P < 0.05$, $\dagger P < 0.01$). **C**, Representative LDPI images of flow recovery in a mouse receiving MSCs vs a mouse receiving media. Red is highest velocity; green, intermediate; and blue, lowest velocity. **D**, Number of mid-thigh collaterals (right) and total collateral cross-sectional area (left) in mice receiving MSCs compared with media or MAECs. $^*P < 0.05$.

numbers appeared to decline, and few cells were seen at day 28. GFP-positive cells were not seen in any calf muscle sections. With the use of β -gal labeling, MSCs were again found distributed widely between muscle fibers (Figure 6A, bottom). However, in conjunction with PECAM staining, multiple adductor muscle sections failed to demonstrate β -gal+ cells incorporated into vessels, suggesting in this model that MSCs did not transdifferentiate into endothelial cells or vascular SMCs.

Local Production of Arteriogenic Cytokines

To confirm that MSCs secreted arteriogenic cytokines in vivo, sections of adductor muscle were examined for colocalization of MSCs and VEGF. Clusters of CFSE+ cells were

seen surrounding VEGF immunostaining (Figure 6B), suggesting local secretion of VEGF from the MSCs. Western blotting and ELISA confirmed significantly higher local adductor muscle production of bFGF and VEGF in those mice receiving MSCs compared with mice receiving media or MAECs (Figure 6C and 6D).

Discussion

To examine the potential role of paracrine mechanisms in augmenting collateral remodeling, we investigated the effects of MSCs because these cells play an important paracrine role in the marrow microenvironment. Previous studies have documented the release by MSCs of a wide array of cytokines that support hematopoietic stem and progenitor cell develop-

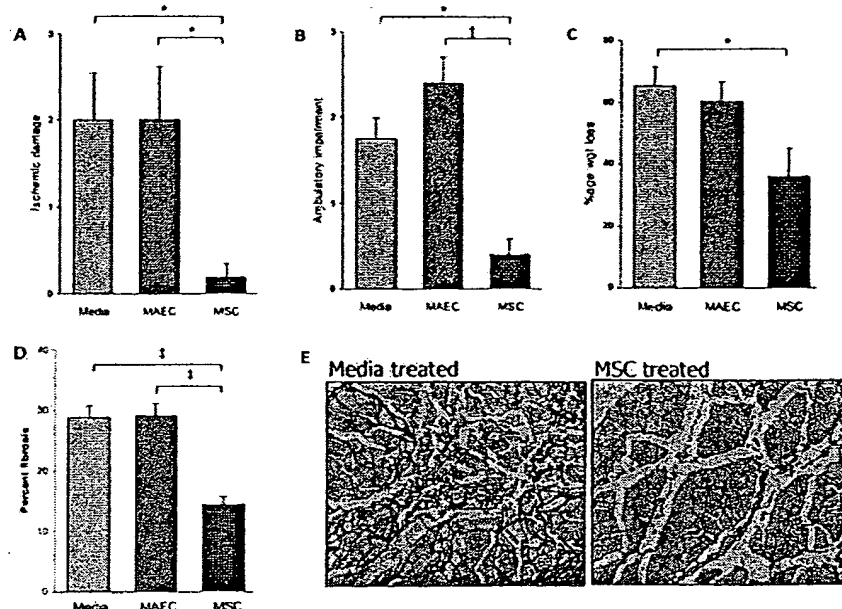


Figure 5. In vivo effects of MSC injection. **A**, Effects of MSC transplantation on ischemic damage ($P < 0.05$). **B**, Effects of MSC transplantation on ambulatory impairment ($P < 0.05$, $\dagger P < 0.01$). **C**, Effects of MSC transplantation on calf atrophy ($P < 0.05$). **D**, Direct injection of MSC into the adductor significantly reduces the degree of calf muscle fibrosis ($\dagger P < 0.001$). **E**, Representative slides of calf muscle after treatment. Control demonstrates fiber atrophy and disarray (yellow) with heavy collagen staining (red). Treatment with MSCs preserves tissue architecture, with significantly less interstitial fibrosis.

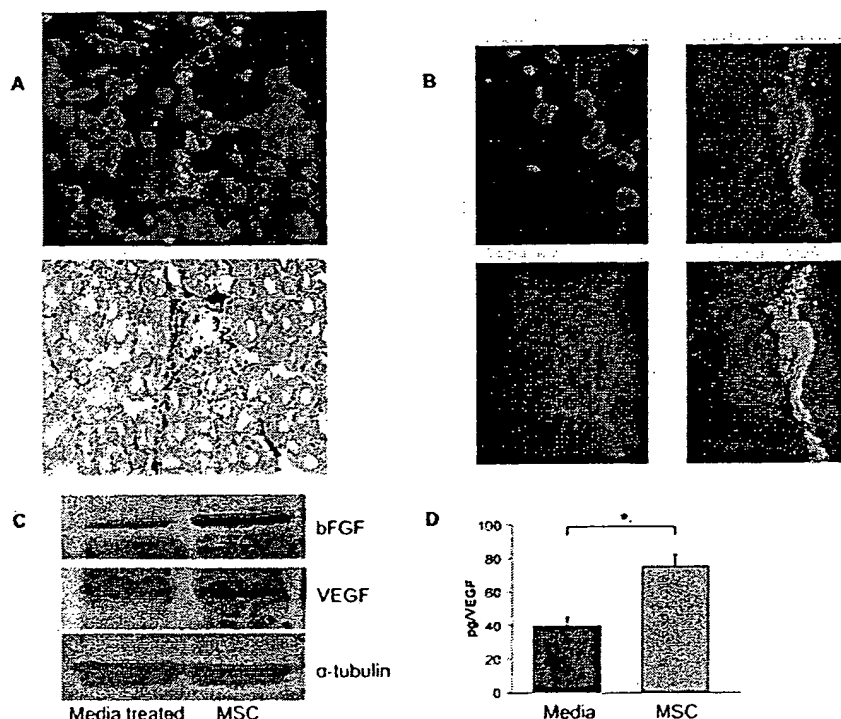


Figure 6. A, Top, $\times 60$ magnification of adductor muscle sections 14 days after injection of GFP-labeled MSCs. 4',6-Diamidino-2-phenylindole (DAPI) stains nuclei blue (in this section, mainly nuclei of skeletal muscle cells). Bottom, Several β -gal-positive MSCs were distributed between muscle fibers. B, Colocalization of CFSE-positive MSCs and VEGF. DAPI staining of nuclei (blue, top left), CFSE-labeled MSCs (green, top right), and VEGF staining (red, bottom left) are shown. CFSE-positive MSCs are surrounded by VEGF staining (mixed colors, bottom right), presumably reflecting secreted VEGF. C, Western blotting (day 7) demonstrates that MSC injection increases local production of VEGF and bFGF compared with control. D, ELISA confirms increased local production of VEGF after MSC injection (* $P < 0.05$).

ment, as well as the secretion of other cytokines that are relevant to augmenting blood flow to ischemic tissue.¹³

In the present study, we demonstrate that MSCs secrete several important arteriogenic cytokines. MCP-1, for example, recruits monocytes to the perivascular compartment, where they orchestrate processes that ultimately lead to collateral vessel growth.¹⁴ Subsequent SMC and endothelial cell proliferation are also crucial in this process, and while VEGF and bFGF enhance proliferation of endothelial cells, bFGF also enhances SMC proliferation. Furthermore, each of these cytokines has been used in a variety of animal models as a single agent to enhance the collateral response to ischemia.¹⁵

We found that injection of MSCs into the adductor muscles of the ischemic hindlimb significantly enhanced perfusion of ischemic tissue and collateral remodeling, lessened tissue damage, and improved limb function. These actions occurred without observable MSC incorporation into vessels. We also found that local production of bFGF and VEGF increased in MSC-injected tissue and documented colocalization of MSCs and VEGF. These results therefore demonstrate that stromal cells can augment collateral remodeling and appear to accomplish this mainly through paracrine pathways.

Previous studies have focused on stromal cell therapy regenerating myocardium when injected into an injured region.^{5,16–18} Although these studies also documented increases in local capillary density, with MSCs found in capillary and arteriolar walls, to our knowledge the present study is the first to demonstrate the potential of stromal cells to augment collateral flow to ischemic tissue through paracrine mechanisms. Of interest, this effect was achieved with injection of the MSCs into tissue proximal to the site of arterial ligation and that manifested no or minimal ischemia.

Several explanations may account for the fact that whereas we demonstrated MSC-derived improvement in collateral function with no evidence of incorporation of stromal cells into mature collaterals, other studies have demonstrated incorporation of bone marrow-derived populations into blood vessels. First, in previous studies demonstrating incorporation of cells into vessels, cells were delivered into ischemic tissue, and incorporation was found to occur in capillaries. The local milieu is likely to be crucial in directing cells to differentiate, and injection into nonischemic tissue may not direct MSCs to incorporate into vessels and to differentiate into endothelial cells. Second, although such cells can incorporate into capillaries or small vessels present in injured, ischemic tissue, it is possible that they do not efficiently incorporate into remodeling collaterals. Third, although vessel wall incorporation of freshly isolated bone marrow mononuclear cells and endothelial progenitor cells has been documented, incorporation of MSCs is less well characterized. MSCs isolated in this study protocol may not represent a population able to differentiate and incorporate through an endothelial or smooth muscle lineage. If this is indeed the case, our data remain consistent with the concept that certain bone marrow-derived populations can enhance collateral remodeling without necessarily incorporating into the vessel wall. Finally, it is possible that small numbers of incorporated cells were simply missed during the section preparation. However, in light of the large increase in flow observed after local MSC injection, it seems unlikely that such a small number of cells could account for this effect.

The concept of paracrine effects mediating at least part of the effects of bone marrow cell therapy is not inconsistent with previous data. Several studies have demonstrated that freshly isolated bone marrow mononuclear cells contain mRNA for VEGF, bFGF, and angiopoietin-1, and local

increases in VEGF protein production after bone marrow mononuclear cell therapy were noted.^{6,19-21} Endothelial progenitor cells were also found to release several cytokines, including VEGF and granulocyte-monocyte colony-stimulating factor.²² Furthermore, injection of human-derived angioblasts into infarcted rat myocardium stimulated host endothelial cells to proliferate, suggesting that these angioblasts may also be a source of proangiogenic factors.²³ Therefore, these data combined with the present study imply that local cytokine release may be an important factor mediating the beneficial arteriogenic effects seen after delivery of bone marrow cells.

Local delivery of MSCs may also cause circulating stem/progenitor cells to home to the region of injury and contribute to healing. MSCs play an important hematopoietic supportive role and have an intimate relationship with stem/progenitor cells in the marrow cavity. In the present study we documented MSC release of several stem/progenitor cell chemokines, including VEGF and MCP-1. Previous studies have documented that MSCs release other stem/progenitor cell chemokines, including hepatocyte growth factor and stem cell-derived factor.²⁴ Therefore, it is highly likely that the collateral enhancing effects of cell therapy are mediated through multiple pathways, including paracrine effects on local vascular cells and chemoattractant effects leading to homing of circulating stem and or progenitor cells.

In addition to the direct therapeutic potential of these cells, the present study demonstrates that MSCs may be used as a vector for gene therapy. MSCs expressed adenoviral transgene product for at least 2 weeks after injection and, unlike fresh mononuclear cells, appeared relatively permissive to adenoviral transduction. This potential was previously exploited in a study demonstrating that MSCs engineered to overexpress interferon- β inhibited the growth of malignant cells in vivo.²⁵

In summary, this study demonstrates that (1) MSCs produce a wide array of arteriogenic cytokines; (2) direct injection of MSCs into a region of forming collaterals improves perfusion and remodeling, lessens tissue damage, and enhances limb function in a mouse model of hindlimb ischemia; and (3) these effects appear to be mediated largely through paracrine mechanisms with local release of arteriogenic cytokines.

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